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(54) Title: ANGIOGENIC HEPARIN-BINDING EPITOPES, PEPTIDE AMPHIPHILES, SELF-ASSEMBLED COMPOSITIONS AND RELATED METHODS OF USE
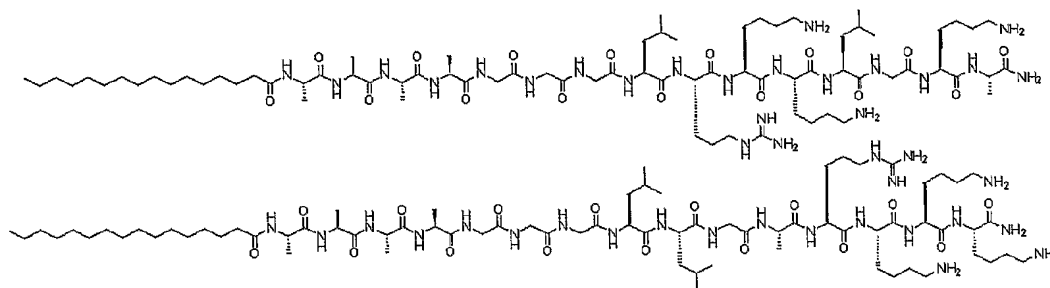
(57) Abstract: Peptide amphiphiles and related compositions comprising sulfated polysaccharides, such as but not limited to sulfated glycosaminoglycans, and methods of use relating to the encapsulation and/or controlled release of angiogenic growth factor(s).

Angiogenic Heparin-Binding Epitopes, Peptide Amphiphiles,
Self-Assembled Compositions and Related Methods of Use

This application claims priority benefit from provisional application serial no.
60/658,503, filed March 4, 2005, the entirety of which is incorporated herein by
reference.

Background of the Invention

Angiogenesis, the process of forming new blood vessels from existing ones, is
essential for normal wound healing, and is well regulated by the body. Inadequate
angiogenesis can give rise to a variety of disease conditions, including chronic skin
wounds and myocardial infarction. Angiogenesis will increasingly become important
for tissue engineering because implanted scaffolds, whether they deliver autologous
cells or recruit host cell infiltration, need to have a blood supply to support the
formation of living tissue. Toward this goal, a concern has been the development of a
biocompatible matrix that can actively promote angiogenesis, with designed chemical
and structural versatility, such that with appropriate modifications it could be used as a
vascularizing scaffold to promote both tissue healing and tissue growth. Further, such a
matrix would also be useful in promoting ischemic wound healing as seen after
myocardial infarction and in chronic skin wounds. The development of and
implementation of such systems have been on-going concerns in the art. However,
various approaches previously taken suggest the need for continued improvement and
provide the impetus toward further effort and innovation.

Brief Description of the Drawings.

Figure 1. Structures of HBPA-1 (top) and HBPA-2 (bottom) amphiphilic
peptide compounds, in accordance with certain embodiments of this invention.

Figures 2A-C. Transmission electron micrographs of heparin triggered bundles of nanofibers of HBPA-2 (2A, scale bar 50 nm) and HBPA-1 (2B, scale bar 40 nm). 2B also shows heparin tagged to gold nanoparticles (black dots) decorating the nanofibers. 2C shows confocal fluorescent micrograph of fluorescein heparin staining bundles of HBPA-1 fibers (scale bar 100 μm).

Figures 3A-G. HBPA-1 and 2 interactions with heparin. 3A and 3B show oscillating rheometry of heparin and base triggered HBPA-1 gels (3A) and HBPA-2 gels (3B). The black curves in both figures are of heparin triggered gels and the grey curves are of base triggered gels with squares representing the elastic modulii and triangles the viscous modulii. The elastic modulii of all the gels are statistically higher than the viscous modulii and further the heparin triggered gels in both cases are statistically higher than that of the base triggered gels (p< 0.05, values represent average and standard deviation). 3C and 3D show circular dichroism spectra of HBPA-1 solution (3C) and HBPA-2 solution (3D) revealing a predominant α helical conformation (grey), changing to predominantly β sheet conformation (black) after heparin is added in both cases. 3E and 3F show the integrated values of the heat change (black dots) and the fit line (line) obtained upon addition of increments of heparin into a solution of HBPA-1 (3E) and HBPA-2 (3F) plotted against the molar ratio of heparin to the HBPAs in order to obtain the respective K_a. Table 3G compares the thermodynamic signature of HBPA-1 and HBPA-2 interaction with heparin. While the ΔG in both cases is similar, ΔH is predominant in SPA heparin interaction indicating an entropically driven reaction while $-T\Delta$S is predominant in HBPA heparin interaction indicating an enthalpically driven reaction.

Figure 4. Slow release of rhodamine-FGF-2 from a network of HBPA-1-heparin gel (gray curve) vs. the more rapid release from a HBPA-1-Na_2HPO_4 gel (black curve)(Bars are standard deviations).

Figures 5A-H. In vitro angiogenesis assay. Fluorescent confocal micrographs of bPAECs stained with Vybrant CFDA in heparin-nucleated HBPA-1 gels with (A) and without the growth factors (B). The black channels are continuous lumina extending in three dimensions (each side of scale grid in (A) is 75 μm and in (B) is 37 μm). Samples

corresponding to HBPA-2 –heparin gels with (C) and without (D) growth factors (scale bars = 80 μm) shows occasional slit like lumen (arrows). Collagen control gels, with growth factors incorporated within the collagen gel (E) shows cells growing with no particular orientation; whereas collagen gels with supplemental heparin (F), with supplemental growth factors (G) and both supplemental heparin and growth factors (H), all show anastomosing networks with occasional slit-like lumina (arrows) (scale bar for C-F = 40 μm).

Figure 6 In vivo ischemic wound healing assay. The epithelial gap measured twelve days after creation of a 6 mm wound on an ischemic rabbit ear. HBPA-1-heparan gels with and without growth factors induced statistically significant wound healing as compared to all other controls (p <0.05, graph represents average and 95% confidence levels).

Summary of the Invention.

In light of the foregoing, it is an object of the present invention to provide a range of amphiphilic peptide compounds, related heparin-bound compositions and/or their use in one or more angiogenic methods, thereby overcoming various deficiencies and shortcomings of the prior art, including those outlined above. It will be understood by those skilled in the art that one or more aspects of this invention can meet certain objectives, while one or more other aspects can meet certain other objectives. Each objective may not apply equally, in all its respects, to every aspect of this invention. As such, the following objects can be viewed in the alternative with respect to any one aspect of this invention.

It can be an object of the present invention to provide a range of structurally diverse amphiphilic peptide compounds interactive with one or more sulfated glycosaminoglycan components, such interaction favorably compared with the prior art with respect to the affinity of such components toward angiogenic growth factors.

It can be another object of the present invention, in conjunction with one or more of the aforementioned compositions, to provide for the activation, binding, delivery and/or release of one or more angiogenic growth factors.

It can be another object of the present invention to provide one or more methods, and compositions useful in conjunction therewith, of inducing angiogenesis, to promote tissue healing and/or growth.

Other objects, features, benefits and advantages of the present invention will be apparent from this summary and the following descriptions of certain embodiments, and will be readily apparent to those skilled in the art having knowledge of various peptide amphiphiles, sulfated polysaccharide bound compositions and/or their use in the promotion of angiogenesis. Such objects, features, benefits and advantages will be apparent from the above as taken into conjunction with the accompanying examples, data, figures and all reasonable inferences to be drawn therefrom, alone or with consideration of the references incorporated herein.

In part, the present invention can be directed to an amphiphilic peptide compound comprising a hydrophobic component and a peptide component. The hydrophobic component can be coupled to the peptide component at, near or about either the C-terminus or the N-terminus of the peptide component. The peptide component can comprise at least one residue capable of non-covalent interaction or binding with a sulfated polysaccharide. Without limitation, such residues can be interactive with or have a non-covalent binding affinity for a sulfated glycosaminoglycan component including but not limited to heparin sulfate, heparan sulfate and combinations thereof. As illustrated elsewhere herein and described more fully in one or more of the references incorporated hereinafter, the hydrophobic component of such a compound can comprise such a moiety ranging from about C_4 or about C_6 to about C_{22} or higher.

Regardless, interactive residues can comprise at least one hydrophobic residue, as can be designated X, such a residue as can be selected from alanine, glycine, leucine, isoleucine, phenylalanine, proline, valine and combinations thereof. Likewise, without limitation as to identity of residue(s) X, the peptide component can comprise at least one basic residue, as can be designated B, including but not limited to arginine, histidine and lysine. In certain embodiments, the interactive residues can comprise a sequence selected from but not limited to XBBBXXBX, XXXXBBBB, XXXXBBB,

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XXXXBB, and XXXXB, wherein X and B can be independently selected from any of the aforementioned hydrophobic and basic residues, respectively. For instance, the peptide components of such compounds can comprise residues comprising a sequence selected from LRKKLGKA and LLGARKKK. Regardless, the peptide component can also comprise one or more bioactive epitope sequences of the sort described below or discussed more fully in one or more of the incorporated references. In certain other embodiments, with or without such a bioactive epitope and without limitation as to interactive residue sequence, the C-terminus of the peptide component can comprise either an amide or a carboxyl moiety.

In part, this invention can also be directed to a composition comprising a sulfated polysaccharide and one or more amphiphilic peptide compounds of the sort described above. Non-covalent interaction of such a sulfated polysaccharide component with an amphiphilic peptide compound can, in an appropriate medium, induce a micellar configuration. For instance, a hydrogel of one or more of the aforementioned peptide components can be induced, in an aqueous medium, by contact with or incorporation of a sulfated glycosaminoglycan. In certain other embodiments, as illustrated below, such compositions can also comprise an angiogenic growth factor. Such growth factors include those as would be understood known or determined by those skilled in the art, representative non-limiting examples of which can be selected from those currently known, and as may later be determined to be, heparin binding or heparan binding growth factors, including but not limited to those designated VEGF and FGF-2, and combinations thereof.

In part, the present invention can also be directed to a method of inducing angiogenesis. Such a method can comprise, without limitation as to order or progression, providing one or more amphiphilic peptide compounds of the sort described above; incorporating therewith a sulfated glycosaminoglycan; and contacting the resulting composition with a cellular medium and/or an angiogenic growth factor. Contact with a cellular medium can be for a time and in an amount of the composition and/or growth factor at least partially sufficient for angiogenesis.

The peptide component of such an amphiphilic compound or a resulting composition can comprise residues comprising a sequence selected from XBBBXXBX, XXXXBBBB, XXXXBBB, XXXXBB, and XXXXB, wherein X can be independently selected from alanine, glycine, leucine, isoleucine, phenylalanine, proline and valine. Likewise, without limitation as to the identity of residue(s) X, residue B can be independently selected from arginine, histidine and lysine. Regardless of sequence, such residues can be interactive with any one or more of the range of known sulfated glycosaminoglycan components, such as but not limited to heparin sulfate, heparan sulfate and combinations thereof. As illustrated elsewhere herein, incorporation of such a glycosaminoglycan component can be used to induce gelation of the peptide compound(s), to provide the resulting composition a micellar configuration. Accordingly, such incorporation and resulting gelation can be effected prior to contact with a cellular medium. In the alternative, an amphiphilic peptide compound can be introduced to or contacted with a cellular medium. Thereafter, incorporation of a glycosaminoglycan component can induce in situ gelation - at, on or within the cellular medium.

In part, this invention can also be directed to a method of using an amphiphilic peptide composition to activate an angiogenic growth factor. Such a method can comprise providing an amphiphilic peptide-sulfated polysaccharide composition of the sort described above; and interacting such a composition with an angiogenic growth factor, as illustrated elsewhere herein to induce angiogenesis in vitro, in vivo, or as would otherwise be recognized by those skilled in the art as indicative of the activation of such growth factors.

In certain embodiments, such interaction can comprise introduction of one or more growth factors to such a composition, either before or after contact between the composition and cellular medium. In certain other in vivo embodiments of such a methodology, interaction can be substantially absent exogenous growth factor, with respect to the cellular medium. As illustrated below, representative of such embodiments, in vivo angiogenesis can be observed, without introduction or addition of an angiogenic growth factor, after cellular contact. Accordingly, various

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embodiments of this methodology can be used to activate an angiogenic growth factor, induce or promote angiogenesis and treat mammalian ischemic tissue.

Detailed Description of Certain Embodiments.

Illustrating certain embodiments of this invention, one or more peptide amphiphile (PA) compounds can be used as a chemical platform to produce a self-assembling, angiogenic scaffold. Such peptide amphiphiles can comprise a hydrophilic peptide head group and a hydrophobic fatty acid tail to induce self-assembly into nanofibers in aqueous solution. For instance, as can be applicable to certain embodiments, a gel or a hydrogel network can be created through utilization of appropriate changes in pH or ionic strength. *See,* Hartgerink, J. D., E. Beniash and S. I. Stupp; "Self-assembly and mineralization of peptide-amphiphile nanofibers." *Science* 294, (2001) 1684-1688, incorporated herein by reference in its entirety.

Alteration of the peptide sequence can be used to impart distinct biological functionalities to the resulting nanofibers. For instance, a peptide amphiphile with a heparin-binding head group can be used because heparin, part of a group of related glycosaminoglycans called heparan sulfate like glycosaminoglycans (HSPGs) that are normally found in the extracellular matrix, are believed to play a role in angiogenesis. HSPGs comprise sulfated glycosaminoglycans including heparin sulfate and its close structural analog heparan sulfate. HSPGs bind to and activate many angiogenic growth factors, in particular–vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2). *See, e.g.,* the following, each of which is incorporated herein in its entirety, Keyt, B. A., L. T. Berleau, H. V. Nguyen, H. Chen, H. Heinsohn, R. Vandlen and N. Ferrara; "The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency." *Journal of Biological Chemistry* 271, (1996) 7788-7795. Herr, A. B., D. M. Ornitz, R. Sasisekharan, G. Venkataraman and G. Waksman; "Heparin-induced self-association of fibroblast growth factor-alpha - evidence for two oligomerization processes." *Journal of Biological Chemistry* 272, (1997) 16382-16389. Schlessinger, J., A. N. Plotnikov, O. A. Ibrahimi, A. V. Eliseenkova, B. K. Yeh, A. Yayon, R. J. Linhardt and M. Mohammadi; "Crystal structure of a ternary fgf-fgfr-heparin complex reveals a dual role for heparin in fgfr

binding and dimerization." *Molecular Cell* 6, (2000) 743-750. This approach imparts versatility to the resulting matrices, as HSPGs are capable of binding and activating many organogenic growth factors across different systems. Various other sulfated polysaccharides can be considered in conjunction with the design of useful peptide sequences. For instance, consistent herewith, residues interactive with carrageenan are incorporated within a peptide component.

Another level of versatility is provided by the peptide amphiphile, itself, since a wide range of peptide epitopes can be incorporated on the periphery of the nanofibers, and judicious design of the molecules can enable co-assembly of multiple PAs with different epitopes into hydrogels. (Niece, K. L., J. D. Hartgerink, J. Donners and S. I. Stupp; "Self-assembly combining two bioactive peptide-amphiphile molecules into nanofibers by electrostatic attraction." *Journal of the American Chemical Society* 125, (2003) 7146-7147, incorporated herein by reference in its entirety.)

In conjunction with the preceding, unique heparin binding sequences can be synthesized, including but not limited to -XBBBXXBX-, where X can be independently selected from hydrophobic amino acid residues and B can be independently selected from basic amino acid residues. The most commonly occurring amino acids in this motif can be determined from a group of naturally occurring heparin-binding proteins. (Cardin, A. D. and H. J. R. Weintraub; "Molecular modeling of protein-glycosaminoglycan interactions." *Arteriosclerosis* 9, (1989) 21-32.) A heparin binding peptide amphiphile (HBPA) of this invention is shown here to self-assemble with the addition of heparin or heparan, leading to formation of a gel. Further, a resulting compositional matrix has the capability to induce endothelial cells sandwiched within it to form highly organized, capillary-like structures with continuous lumen in three dimension; and, a resulting matrix with heparan has been shown to significantly improve ischemic wound healing even without growth factors-something not observed in the literature with any other type of matrix.

In one respect, compounds of this invention can comprise a peptide amphiphile incorporating such a binding sequence; that is, any heparin-binding peptide amphiphile of the form:

(hydrophobe) – (spacer) – XBBBXXBX- (terminus)

where the hydrophobe component is any saturated or unsaturated alkane or other hydrophobic moiety, (spacer) is an optional component comprising an arbitrary amino acid sequence, X can be independently selected from alanine, glycine, leucine, isoleucine, phenylalanine, proline and valine, and B can be independently selected from arginine, histidine, and lysine and (terminus) is an amide or carboxyl terminated amino acid residue or sequence or other epitope which may be known or determined to be bioactive, such as but not limited to RGD, IKVAV, and biotin. Various other epitopes are known in the art and/or as described in one or more of the incorporated references.

Without limitation, one of the HBPA compounds of this invention can comprise a fatty acid, e.g. a palmitic acid, moiety or otherwise hydrophobic component covalently linked or coupled to a peptide sequence such as AAAAGGGLRKKLGKA, with a terminal alanine residue optionally amide terminated. The presence of a hydrophobe induces self-assembly into nanofibers in aqueous solutions when triggered with appropriate stimuli, such as the addition of heparin. Further, appropriate concentrations of the HBPA with the addition of heparin, heparan or similar highly charged polymers causes the formation of a self-supporting hydrogel, due to the entanglement of bundles of nanofibers. This HBPA-heparin interaction is non-covalent, which is an improvement over current covalently bound heparin matrices as non-covalent interaction simulates biological interaction of heparin to extra-cellular matrix. The non-covalent interaction also allows the heparin to bind and activate angiogenic heparin-binding growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2), and control their release from the matrix.

In particular, certain embodiments of this invention can comprise a heparin-binding peptide comprising the amino acid sequence, –LRKKLGKA– which is both novel and potentially useful for covalent or non-covalent attachment to a wide range of bioactive polymers, scaffolds and tissue or cell culture substrates where binding of heparin or heparin-like polymers is desired. Further, since the bulk of the non-covalent

interaction between heparin and the heparin binding peptide amphiphile can be explained at least in part by electrostatic attraction, other, related sequences have also been prepared in the form of peptide amphiphiles, following the general format of (hydrophobe) – (spacer) – XXXXBBBB- (terminus); (hydrophobes) – (spacer) – XXXXBBB- (terminus); (hydrophobe) – (spacer) – XXXXBB- (terminus) and (hydrophobe) – (spacer) – XXXXB- (terminus) where the hydrophobe component, the optional (spacer) component, X, B and (terminus) are as defined above.

Specifically, one such peptide amphiphile includes but is not limited to the structure: palmitoyl-AAAAGGGLLGARKKK with an amide terminus. Regardless, the peptide component of amphiphilic compounds useful with this invention is limited only by capacity to bind and/or utilize heparin, and/or functionally equivalent heparin derivatives or analogs thereof, according to or consistent with the descriptions herein or as would be inferred by those skilled in the art made aware of this invention.

Regardless of heparin-binding capability, the peptide amphiphiles of this invention can comprise a peptide component of varied length or sequence depending upon desired flexibility, charge and/or capacity for intermolecular interaction or binding enroute to nanofiber formation. A hydrophobic component of such compounds can also be varied (e.g., moieties ranging from about C_4 or about C_6 to greater than about C_{22} or higher alkyl or substituted alkyl, saturated or unsaturated, etc.), such components limited only by resulting amphiphilic character and effect on compositions or assemblies of such compounds.

Various peptide amphiphile compounds used in conjunction with the present invention, with consideration of any one or more of the preceding considerations, can be synthesized using preparatory techniques well-known to those skilled in the art, including those disclosed in co-pending applications serial nos. 10/294,114 filed November 14, 2002 (International Publication No. WO 03/054146) and 10/368,517 filed February 18, 2003 (International Publication No. WO 03/070749), each of which are incorporated herein by reference in their entirety, and modifications of those techniques known in the literature and as referenced elsewhere herein. The synthetic schemes set forth in such references and co-pending applications may be applied to the

present invention. Peptide amphiphiles may be fully protonated, partially protonated, or as acid or basic addition salts. Generally, such peptide amphiphiles can be prepared using standard solid-phase peptide chemistry including addition of a hydrophobic tail or component at or near the N-terminus of the peptide component. Modifications of such synthetic techniques can be made as would be known to those skilled in the art and aware of this invention, such as by using procedures and the corresponding peptide amphiphile moieties, compounds, related compositions, and configuration or assemblies described in co-pending application serial nos. 11/005,314 and 11/005,552 filed on December 6, 2004 (International Publication Nos. WO 05/056576 and WO 05/056039, respectively), each of which is incorporated herein by reference in its entirety.

An HBPA compound can comprise, for example, a fatty acid tail derived from palmitic acid, a linker peptide of four alanines and three glycines and a novel heparin binding peptide head group containing the amide terminated sequence LRKKLGKA (referred to as HBPA-1 henceforth) or the amide terminated sequence LLGARKKK (referred to as HBPA-2 henceforth) (see Figure 1). Both HBPA-1 and -2 are readily soluble in water, and self-assemble to form bundles of nanofibers in solution. At concentrations above six millimolar of the two HBPAs, addition of heparin or heparan triggered gel formation. These bundles of nanofibers were visualized by transmission electron microscopy (TEM) shown in Figure 2A, with heparin tagged gold particles seen decorating HBPA-1 nanofibers (Figure 2B). Further, fluorescent confocal microscopy showed bundles of HBPA-1 fibers to be stained by heparin tagged to fluorescein, as shown in Figure 2C. Frequency sweep oscillating rheology revealed viscoelastic gel-like behavior for these materials, with both the storage (G') and loss (G") modulus largely independent of the angular frequency and G' consistently higher than G" (see Figure 3A and 3B). The HBPAs also gelled both at elevated pH (base triggered) and with the addition of disodium hydrogen phosphate. Further, the elastic modulii of the heparin triggered gels was statistically higher in both cases as compared to the respective base triggered gels indicating increased stiffness (Figures 3A and 3B).

Circular dichroism (CD) spectroscopy of HBPAs showed a CD signature with predominant alpha helical content. This changed with the addition of heparin into a signature suggestive of beta sheet formation with typical negative and positive maxima at 218 nm and 192 nm respectively (see Figure 3C and 3D). Isothermal titration calorimetry was used to titrate increments of heparin independently into both the HBPAs and measured the heat released upon binding as a function of the molar ratio. The data obtained was integrated and fitted to a nonlinear function as previously described (Fromm, J. R., et al, "Differences in the Interaction of Heparin with Arginine and Lysine and the Importance of These Basic-Amino-Acids in the Binding of Heparin to Acidic Fibroblast Growth-Factor" *Arch. Biochem. Biophys.* 323 (1997) 279) to obtain an association constant of 10^7 in both cases (see Figure 3E and 3F). Despite similarity in their binding constants, the binding interaction of HBPA-1 and HBPA-2 were energetically very different. The HBPA-1 and heparin interaction appears to have been predominantly driven by entropic changes whereas the HBPA-2- heparin interaction was predominantly enthalpic (Table 3G). Such results can be explained with reference to their respective structures. HBPA-1 has hydrophobic residues on the periphery of its peptide chain and the increase in entropy is possibly due to displacement of solvent water molecules from these residues upon heparin interaction. HBPA-2, on the other hand, has the charged basic residues on the periphery leading to strong electrostatic forces with the negatively charged heparin, and hence the predominance of enthalpic factors in their interaction.

A release profile of fibroblast growth factor-2 (FGF-2) from HBPA-1-heparin gel was determined, illustrating another aspect of this invention. FGF-2 covalently linked to rhodamine (ex/em maxima at 544/576 nm) was incorporated into HBPA-1 hydrogels prepared with either the addition of heparin or disodium hydrogen phosphate. The release media was exchanged and stored at a series of time points. The passive cumulative release profiles of the FGF-2 rhodamine revealed that, in the absence of heparin, 34.1 % of the FGF-2 was released from the gel within the first five minutes and 98.3% was released by day 10. The presence of heparin reduced the rate and the absolute release of the FGF-2 to a total of 57.1% by day 10 (see Figure 4).

To demonstrate in vitro angiogenesis, bovine pulmonary artery endothelial cells (bPAEC) were grown to confluence on top of a layer of both types of HBPA-heparin gel and then sandwiched by the application of another layer of the same gel in an 8-well chambered coverslip. Some gels had a combination of VEGF and FGF-2 incorporated within them. Four controls were used: bPAECs sandwiched within two layers of type I collagen gels to which no supplemental heparin or growth factors were added; supplemental heparin alone; growth factors alone; or both heparin and growth factors added at each media change. The bPAECs grew in sheets and showed branched anastomosing networks as early as one day after the addition of the second layer in the HBPA-1-heparin gels with growth factors. This organization continued and by day 7 showed formation of organized tubular structures with continuous lumens penetrating through the thickness of the gel (see Figure 5A). The HBPA-1-heparin gels without growth factor started showing some branching later at day 3. At day 7, these gels appeared to have fewer tubules than the ones seen in the HBPA-1-heparin gels with growth factors, but the individual tubules in both types of gels showed remarkable similarity (Figure 5B). In the case of the HBPA-2 heparin gels, the cells grew in sheets in three dimensions with occasional slit like lumens and rare tubular structures seen at the end of ten days in gels with and without growth factors (Figure 5C and D). The collagen gels with no supplemental heparin or growth factors showed the presence of bPAECs growing throughout the gels with no particular organization. The three types of gels with supplemental heparin, growth factors or both showed the presence of branched anastomosing networks in some of the areas. None showed the formation of organized tubular structures with continuous lumen (Figures 5E-H).

Finally, in order to demonstrate the functional efficacy of such a composition and matrix configuration in vivo, a rabbit ear wound healing model was chosen. (*See, e.g.,* Ahn ST, Mustoe TA. "Effects of ischemia on ulcer wound healing: a new model in the rabbit ear." *Ann Plast Surg.*24 (1990) 17-23, the entirety of which is incorporated herein by reference.) This is a well-established model wherein ischemia is induced surgically by tying off two of the three arteries which supply the normal rabbit ear and interrupting skin circulation circumferentially at the ear base. Then, four

wounds are created on the ventral aspect of the ear using a circular 6 mm biopsy punch upto and including the perichondrium. The desired materials in this case HBPA-1 heparan gel with and without the growth factors (VEGF and FGF-2) as the case may be are applied and the wound is covered with a polyurethane film dressing and followed up for twelve days. At the end of twelve days, the animals are euthanized and the wounds are harvested using a through and through 7 mm biopsy around the wound. The samples are analyzed for histological evidence of wound healing. This healing process can be quantified by measuring the epithelial gap between the healing edges in a bisected wound. Four control materials were also used namely HBPA-1 with growth factors, heparan with growth factors, growth factors alone and a buffer solution alone (the solvent for the above materials).

Analyzing the wound edge results, it was found that the HBPA-1- heparan gels induced statistically significantly higher wound healing than any of the controls. The presence of exagenous or introduced growth factors did not seem to be necessary to affect the ability of the matrix to induce wound healing (see Figure 6). Induced wound healing in ischemic wounds without the use of growth factors has not been previously reported. Without limitation to any one theory or mode of operation such observations may be due to the ability of the heparan in the composition and resulting matrix configuration to recruit and activate endogenous growth factors found locally within the cellular medium.

Heparin and heparan are important promoters of angiogenesis due to their ability to bind and activate angiogenic growth factors. Other studies have used heparin to release angiogenic growth factors by covalently binding it to a matrix, physically trapping it within a matrix or by coating the surface of a matrix with heparin. In contrast to the art, this invention incorporated heparin and or heparan non-covalently, using a consensus heparin-binding sequence on a peptide amphiphile (HBPA), to form a hydrogel with the potential to recruit, activate and/or deliver growth factors to cells in a way that mimics the function of heparin in the extracellular matrix.

The self-assembly of other peptide amphiphile molecules into nanofibers that entangle to form gels has been previously described. See, e.g., Hartgerink, J. D.,

E. Beniash and S. I. Stupp; "Peptide-amphiphile nanofibers: A versatile scaffold for the preparation of self-assembling materials." *Proceedings of the National Academy of Sciences of the United States of America 99*, (2002) *5133-5138*. Briefly, it is believed, without limitation to any one theory or mode of operation, that when the pH of the solution is acidic, the HBPAs have a net positive charge that inhibits self-assembly through electrostatically repulsion. As the pH of the solution is raised, the positive charges are neutralized, facilitating aggregation through hydrophobic collapse and the formation of a hydrogen-bonded peptide secondary structure. Gel formation occurs due to entanglement of nanofibers and requires an appropriate concentration of the HBPA. Simple inorganic counter ions have also been shown to promote this self-assembly and gel formation, presumably due to a similar charge-shielding role. Here, self-assembly is observed either with addition of inorganic anions from Na_2HPO_4 or with complex polymeric anions– the glycosaminoglycans, heparin sulfate and heparan sulfate. Heparin-triggered self-assembly and gel formation is interesting, as (1) it is the first described instance of a polymeric substance triggering supra-molecular self-assembly, and (2) because the peptide component was specifically designed to bind to such glycosaminoglycans. Heparin can be considered as not only performing a simple charge shielding role, but as also involved in forming noncovalent crosslinks between nanofibers. As such, heparin could bind to multiple HBPA molecules, of differing hydrophobic components or residue sequences, and thus template a mixed supramolecular self-assembly.

The interactions of the HBPAs with the heparin are further confirmed by CD spectroscopy and isothermal calorimetry. The binding constant obtained by ITC of 100 nM is indicative of strong binding and is comparable to that obtained between other synthetic heparin binding peptides and heparin. At the same time, this is two orders of magnitude weaker than the binding constant of heparin to a heparin binding growth factor like FGF-2, and hence heparin containing hydrogels are able to retain FGF-2 for longer periods of time than the HBPA alone and slow its release from the hydrogel.

The cell sandwich in-vitro assays showed the presence of highly organized, tubular structures with continuous lumen penetrating through the thickness of the HBPA-1-heparin gels. The structures seen closely resembled in vivo capillary networks with a degree of organization not previously reported. This behavior was seen only in the HBPA-1-heparin gels. The HBPA-1-heparin gels with growth factors were observed to organize sooner and over larger areas than the gels without growth factors. Though the presence of added growth factors induced earlier anastomosis, the gels without growth factors also exhibit similar organization, possibly due to the ability of the noncovalently bound heparin in the gel to recruit and activate growth factors from the serum and those synthesized by the cells themselves. This would explain the qualitative similarity of the tubular processes in the HBPA-1-heparin gels both with and without growth factors and the delay in organization of the cells in the HBPA-1-heparin gels without growth factors. It can be postulated that formation of bundles of nanofibers non-covalently exhibiting heparin on its surface optimizes the bioactivity of heparin for this particular application. In contrast, the HBPA-2 heparin gels show occasional discontinuous slit-like lumen similar to the control collagen gels. This could be because the presence of the consensus format in the first case optimizes this particular bioactivity of heparin. Consensus heparin-binding sequences of naturally occurring heparin-binding proteins are thought to form a positively charged alpha turn of 20 A around the negatively charged repeat unit of heparin. (Margalit, H., N. Fischer and S. A. Bensasson; "Comparative-analysis of structurally defined heparin-binding sequences reveals a distinct spatial-distribution of basic residues." *Journal of Biological Chemistry* 268, (1993) *19228-19231*.)

Finally, in vivo models of ischemic wound healing on rabbit ears shows that HBPA-1 heparan gels even without growth factors significantly induces wound healing which would result from improved angiogenesis locally. Of striking note is the fact that this wound healing was accomplished even without the angiogenic growth factors. This is probably due to the presence of endogenous growth factors at the wound site which is being recruited and activated by the HBPA-1 heparan matrix. This is a completely novel result and in fact previous studies have shown a partial improvement

with wound healing in this model only with the use of micrograms of growth factors (Corral CJ, Siddiqui A, Wu L, Farrell CL, Lyons D, Mustoe TA. "Vascular endothelial growth factor is more important than basic fibroblastic growth factor during ischemic wound healing." *Arch Surg. 134* (1999), *200-205)*.

Accordingly, the present invention can provide a novel class of peptide amphiphile biomolecules that self-assemble and bind noncovalently to heparin, heparan, and other sulfated glycosaminoglycans, giving rise to an angiogenic hydrogel that was characterized in vitro and in vivo. Such compounds can be triggered with a polymeric substance, such as an HSGAG, to self-assemble from solution into a gel. Biologically, an HBPA-heparin/heparan gel, representative of other compositions and configurational matrices of this invention, has the unique ability to induce endothelial cells to form highly organized capillary-like tubules with continuous lumen in three dimensions in culture and most important of inducing ischemic wound healing without exogenous growth factors.

Examples of the Invention.

The following non-limiting examples and data illustrate various aspects and features relating to the amphiphile compounds, nanofibers, gels, compositions and/or methods of the present invention, including the self-assembly of heparin-binding peptide amphiphiles and corresponding delivery of heparin, heparan and/or related growth factors, as are available through the methodologies described herein. In comparison with the prior art, the present methods, compounds and compositions provide results and data which are surprising, unexpected and contrary thereto. While the utility of this invention is illustrated through the use of several amphiphilic compounds and components thereof, it will be understood by those skilled in the art that comparable results are obtainable with various other amphiphile compounds and/or components, as are commensurate with the scope of this invention.

Example 1

HBPA gel formation. All reagents were purchased from Fisher and used as received unless otherwise specified. HBPAs were synthesized using methods described in the aforementioned incorporated references. Various other amphiphilic

peptide compositions, in accordance with this invention, comprising other residues and /or hydrophobic components can be prepared as also described therein. Briefly, the peptide was constructed on a Rink amide resin using an automated solid phase peptide synthesizer (Applied Biosystems- 733A) with appropriately protected amino acids (Novabiochem) for standard fluorenylmethoxycarbonyl (Fmoc) chemistry. The N-terminus of the peptide was capped with palmitic acid using an alkylation reaction, followed by deprotection and cleavage of the HBPA from the resin using trifluoracetic acid (TFA), water and trisiopropylsilane. TFA was removed by rotary evaporation and triturated the HBPA product using cold diethyl ether, which was then filtered and vacuum dried. The molecular weight of the HBPA was characterized by electrospray ionization mass spectrometry. The HBPA was solubilized in 1 M hydrochloric acid at room temperature for one hour and then subsequently lyophilized it to decrease the residual TFA counter ions and replace them with chloride ions. The HBPA was resolubilized at 30 mg/mL at pH 7.4 (unless otherwise specified) in de-ionized water using 1 M sodium hydroxide as needed. The HBPA gels were formed by mixing equal volumes of the HBPA solution made as above and the gel trigger – either heparin sodium or heparan sodium Sigma) in concentrations of 20 mg/mL (to obtain a stoichiometry of 1: 1.84 for HBPA: heparin/heparan) or disodium hydrogen phosphate in solution in concentration of 11 mg/ml – to obtain a final product of 1.5 w/v % HBPA gels. Whenever lower weight percent gels were made, the heparin, heparan and the phosphate were scaled down appropriately to maintain the stoichiometry.

<u>Example 2</u>

<u>Characterization of the self-assembly</u>. Heparin-gold stained HBPA samples were prepared for transmission electron microscopy (TEM) as previously described. (Sanantonio, J. D., A. D. Lander, M. J. Karnovsky and H. S. Slayter; "Mapping the heparin-binding sites on type-I collagen monomers and fibrils." *Journal of Cell Biology* 125, (1994) *1179-1188*.) Briefly, a holey carbon coated copper grid was dipped twice in solutions of HBPA-1 (0.1 w/v % in water) for 20 s, stained with colloidal 10 nm gold-tagged heparin-albumin solution diluted 1:20 in the recommended buffer (Sigma) for 30 min. at 4 °C, fixed in 4 v/v % formaldehyde

(Sigma) in phosphate buffered saline (PBS- Gibco) at room temperature for 20 min. and then counter-stained in 2 w/v % uranyl acetate for 45 minutes at room temperature with two washes in 0.1 M cacodylate buffer with 0.5 w/v % bovine serum albumin and 0.05 v/v % Tween 20 between steps (Sigma). In the case of HBPA-2, a holey carbon coated copper grid was dipped twice in a 1% HBPA-2-heparin gel suspension for 20 s and then stained in phosphotungstic acid (Sigma) at room temperature. TEM was performed on a Hitachi 8100 microscope at an accelerating voltage of 200 kV. Confocal fluorescent microscopy was performed by mixing 10 μL each of a 0.04 w/v % HBPA-1 in water solution and 0.03 w/v % in water of fluorescein-heparin (Sigma) solution and imaging with a Leica laser confocal scanning microscope (DM IRE2). The images were analyzed using the Leica LCS imaging software. A Paar Physica MCR300 rheometer with a stainless steel parallel plate of 20 mm was used to perform oscillating rheology experiments on gels prepared in situ by mixing 80 μL 2 w/v % HBPAs in water and either 1 mg of heparin or 0.5 mg of disodium hydrogen phosphate in 80 μL of water or adding 80 μL of 0.25 M NaOH and maintained temperature at 22 °C. A frequency sweep experiment was performed at 3 % strain with a ten-minute wait time (both determined by independently performing an amplitude sweep and a time strain experiment) to obtain 17 data points between angular frequencies of 0.1 to 10 rad/s. CD spectra were collected, on a Jasco J-715 CD spectrometer using a 0.1 cm path length quartz cuvette, from four samples: blank control, 0.105 mg of HBPA-1 or HBPA-2, 0.07 mg of heparin and a mix of 0.105 mg of the two HBPAs separately and 0.07 mg heparin each in 350 μL of water at pH 7. Isothermal calorimetry (Microcal-ITC) was performed by titrating heparin in 4 μL aliquots from a stock solution of 101.5 μg/mL solution into a 40.1 μg/ ml HBPA-1 or-2 solution (all solutions in water). The same amount of heparin was titrated into a blank solution to obtain background values. The raw data was obtained in terms of the heat released by the binding between the two versus their molar ratio to the data was integrated and fit to a curve for a single type of binding site to obtain a binding constant as described previously, referenced above.

Example 3

Release profile of FGF-2 from HBPA-1-heparin gel. FGF-2 (Peprotech) was
covalently linked to N-hydroxysuccinimide- rhodamine by means of an ester linkage
using a commercially available rhodamine protein labeling kit (Pierce Biotechnology),
adding 12.5 ng of this FGF-2 rhodamine to a 100 μl solution of either 20 mg/ml
heparin in water or 11 mg/ml disodium hydrogen phosphate. These solutions were
added to a 100 μl solution of 3 w/v % HBPA-1 solution in water to respectively obtain
HBPA-1-heparin or HBPA-1-phosphate gels with FGF-2 rhodamine. The gels were
covered with 100 μl water and incubated at 37 °C in an incubator (5 % CO_2) and
changed initially at 5 minutes and then subsequently every day for 10 days. The
changed water was collected and analyzed using a Gemini EM fluorescence plate
reader (ex/em maxima 544/576 nm). The fluorescence of an aliquot of the original
FGF-2 in heparin or phosphate solution was measured and this value was used to
obtain the percentage released.

Example 4

In-vitro angiogenesis assay. PAEC were grown to passage 14 or 15 in phenol
red free Dulbecco's modified Eagle medium with 20% v/v fetal bovine serum, 1% v/v
penicillin-streptomycin, 2% v/v L-glutamine and 1 mM each of sodium pyruvate and
modified Eagle medium amino acids (the serum was obtained from Hyclone while the
media and other additives from Gibco). The freeze media was made by adding 5 v/v %
dimethyl sulfoxide (Sigma) to the above media. The cells were grown in cell culture
incubators at 37 C with 5% CO_2. The sandwich gels were made in 8-well chambered
cover slip (Nalge Nunc) containers. The first layer of the HBPA-heparin gels was
created by mixing 100 μl of 30 mg/ml of HBPA-1 or -2 in water at pH 7 with 100 μl of
20 mg/ml heparin in the above cell culture media with or without 12.5 ng each (to give
a total concentration in the well of 31.25 ng/ml) of FGF-2 and VEGF (both from
Peprotech). 200 μl 3 w/v % collagen gels were made using type I rat tail collagen
(Roche), which was gelled in a base chamber and then equilibrated with the above
media to obtain a pH of 7.4. The gel was allowed to set by leaving it at room
temperature overnight. Subsequently, 750,000 bPAECs per well were plated in culture

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media and followed up with alternate day media changes in the incubator until cells grew to confluence through the thickness of the gel (usually by day 5). Excess media was removed and the second layer of gel was added on top of the cell layer exactly as before. The collagen gels were made in a separate 8 well chamber slide and then placed on top of the cell layer after pH equilibration as before. After a half hour wait at room temperature, media was added and the wells were incubated at 37 C and then changed media every alternate day. Supplemental heparin was added with or without the growth factors at the same concentrations as above to the specifically defined collagen gel controls. We did not supplement either kind of the HBPA-heparin gels with heparin or growth factors in the media. Hence the only source of supplemental growth factors for both the HBPA-heparin gels with growth factors was from the two gel layers. The cell cultures were observed daily using light microscopy. At day 7, the cells were stained with a fluorescein-based cell tracer (Vybrant CFDA SE cell tracer-Molecular Probes) at 20 μM concentration and imaged them using a Leica laser confocal scanning microscope (DM IRE2) to obtain a z-series through the gels. Volocity and NIH ImageJ software were used for 3-D rendering of the z-series images.

<div align="center">Example 5</div>

Rabbit ear ischemic wound healing assay. An assay was used to measure the ability of the matrix to induce wound healing in an ischemic area (Ahn ST, Mustoe TA. "Effects of ischemia on ulcer wound healing: a new model in the rabbit ear." *Ann Plast Surg.24* (1990) *17-23*)). Protocols were approved by Northwestern's Animal Care and Usage Committee. Animals were anesthetized with ketamine and xylazine and a sterile surgical incision was made 1 cm distal to the root of the ear. The central and rostral arteries were identified, ligated with 4-0 ethilon and interrupted taking care to leave the respective veins untouched. The incision was extended circumferentially around the base of the ear interrupting dermal circulation leaving the small caudal artery as the only source of blood supply to the ears and then sutured close. On the ventral surface, a 6 mm biopsy punch was used to create four circular wounds upto and including the perichondrium leaving the bare cartilage as the wound base. The necessary materials were applied with the HBPA-heparan being gelled in situ where

specified. The wounds were covered with a thin polyurethane wound dressing (Tegaderm ™) and animals were given appropriate post-operative analgesia. The animals were housed for twelve days in the appropriate facility. At the end of twelve days, they were anesthetized and then euthanized with intra cardiac Euthasol ™ followed by surgical induction of pneumothorax to confirm euthanasia. The wounds were harvested with a 1mm cuff of normal tissue using a 7 mm biopsy punch going through to the dorsal skin. These wounds were placed in buffered formalin, fixed, paraffin embedded and stained by Masson's trichrome after bisecting. The gap between the leading edge of the epithelium was measured in each wound in order to quantify wound healing with a measurement of zero indicating complete healing. The results were aggregated and statistically analyzed using a two sample t test assuming unequal variances.

<div align="center">*   *   *</div>

While the principals of this invention have been described in connection with specific embodiments, it should be understood clearly that these descriptions are added only by way of example and are not intended to limit, in any way, the scope of this invention. For instance, certain embodiments have been described as providing a compositional matrix that can bind and control delivery of certain angiogenic growth factors to promote capillary-like structures with a degree of endothelial cell organization not previously reported. However, such a vascularizing matrix can also be used for the controlled delivery and release of various other growth factors. Likewise, such a composition or matrix can be formed in situ upon introduction or injection of liquid precursor compounds or components into a cellular medium.

We claim:

1.   An amphiphilic peptide compound comprising a hydrophobic component and a peptide component, said hydrophobic component coupled to said peptide component at one of the C-terminus and the N-terminus thereof, said peptide component comprising residues capable of non-covalent interaction with a sulfated polysaccharide.

2.   The compound of Claim 1 wherein said peptide component comprises residues interactive with a sulfated glycosaminoglycan selected from heparin sulfate, heparan sulfate and combinations thereof.

3.   The compound of Claim 1 wherein said hydrophobic component comprises an alkyl moiety ranging from about C6 to about C22.

4.   The compound of Claim 1 wherein said interactive residues comprise at least one hydrophobic residue (X) selected from alanine, glycine, leucine, isoleucine, phenylalanine, proline, valine and combinations thereof, and at least one basic residue (B) selected from arginine, histidine and lysine.

5.   The compound of Claim 4 wherein said interactive residues comprise a sequence selected from XBBBXXBX, XXXXBBBB, XXXXBBB, XXXXBB, and XXXXB, wherein X and B are each independently selected from said hydrophobic residues and said basic residues.

6.   The compound of Claim 5 wherein said interactive residues comprise a sequence selected from LRKKLGKA and LLGARKKK.

7.   The compound of Claim 5 wherein said peptide component comprises a moiety selected from a C-terminus amide, a bioactive epitope sequence and combinations thereof.

8.   The compound of Claim 6 in composition with a sulfated polysaccharide, said composition comprising a micellar configuration.

9.   The compound of Claim 8, said composition wherein said polysaccharide is a sulfated glycosaminoglycan selected from heparin sulfate, heparan sulfate and combinations thereof.

10.   The compound of Claim 9, said compositions interactive with an angiogenic growth factor.

11.   The compound of Claim 10, said growth factor selected from a heparin binding growth factor, a heparan binding growth factor and combinations thereof.

12.   A composition comprising a sulfated polysaccharide and an amphiphilic peptide comprising a hydrophobic component and a peptide component, said hydrophobic component coupled to said peptide component at one of the C-terminus and the N-terminus thereof, said peptide component comprising residues capable of non-covalent interaction with said polysaccharide, said composition comprising a micellar configuration.

13.   The composition of Claim 12 wherein said polysaccharide is a sulfated glycosaminoglycan selected from heparin sulfate, heparan sulfate and combinations thereof.

14.   The composition of Claim 12 wherein said interactive residues comprise a sequence selected from XBBBXXBX, XXXXBBBB, XXXXBBB, XXXXBB, and XXXXB, wherein X is independently selected from alanine, glycine, leucine, isoleucine, phenylalanine, proline and valine, and B is independently selected from arginine, histidine and lysine.

15.   The composition of Claim 14 comprising an angiogenic growth factor selected from a heparin binding growth factor, a heparan binding growth factor and combinations thereof.

16.   The composition of Claim 15 wherein said factor is selected from VEGF and FGF-2.

17.   The composition of Claim 16 in contact with endothelial cells.

18.   The composition of Claim 17 wherein said interactive residues comprise a sequence selected from LRKKLGKA and LLGARKKK.

19.   The composition of Claim 14 in contact with a mammalian ischemic skin wound.

20.   The composition of Claim 19 wherein said interactive residues comprise a sequence selected from LRKKLGKA and LLGARKKK.

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21.   A method of using an amphiphilic peptide composition to activate an angiogenic growth factor, said method comprising:

providing an amphiphilic peptide compound comprising a hydrophobic component and a peptide component, said hydrophobic component coupled to said peptide component at one of the C-terminus and the N-terminus thereof, said peptide component comprising residues capable of non-covalent interaction with a sulfated glycosaminoglycan;

incorporating a sulfated glycosaminoglycan with said peptide compound, said glycosaminoglycan selected from heparin sulfate, heparan sulfate and combinations thereof; and

interacting said peptide composition with an angiogenic growth factor.

22.   The method of Claim 21 wherein said interactive residues comprise a sequence selected from XBBBXXBX, XXXXBBBB, XXXXBBB, XXXXBB, and XXXXB, wherein X is independently selected from alanine, glycine, leucine, isoleucine, phenylalanine, proline and valine, and B is independently selected from arginine, histidine and lysine.

23.   The method of Claim 22 wherein said interaction is in vivo.

24.   The method of Claim 23 wherein said interaction is substantially absent exogenous growth factor.

25.   The method of Claim 24 wherein said interactive residues comprise a sequence selected from LRKKLGKA and LLGARKKK.

26.   The method of Claim 23 contacting mammalian ischemic tissue.

27.   A method of inducing angiogenesis, said method comprising:

providing an amphiphilic peptide compound of Claim 1;

incorporating a sulfated polysaccharide with said peptide compound; and

contacting said composition with a cellular medium and an angiogenic growth factor, said contact with said medium for a time and in an amount of at least one of said composition and said growth factor at least partially sufficient for angiogenesis.

28.   The method of Claim 27 wherein said interactive residues comprise a sequence selected from XBBBXXBX, XXXXBBBB, XXXXBBB, XXXXBB, and XXXXB, wherein X is independently selected from alanine, glycine, leucine, isoleucine, phenylalanine, proline and valine, and B is independently selected from arginine, histidine and lysine; and said polysaccharide is a sulfated glycosaminoglycan selected from heparin sulfate, heparan sulfate and combinations thereof.

29.   The method of Claim 28 wherein said growth factor is exogenous to said medium.

30.   The method of Claim 28 contacting said peptide compound and said medium, prior to incorporation of said glycosaminoglycan.

31.   The method of Claim 30 wherein said contact is in vivo.

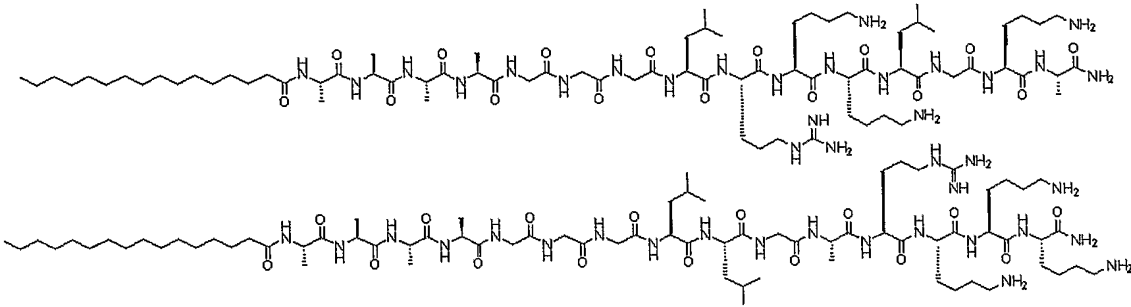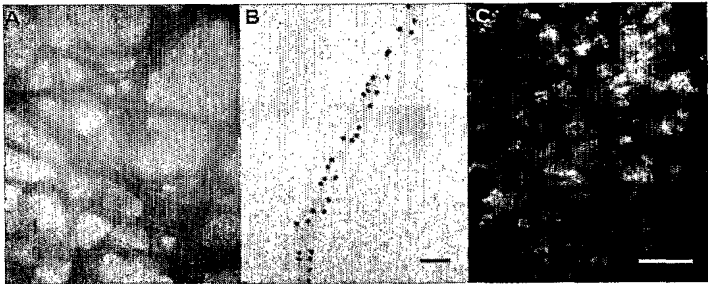32.   The method of Claim 31 contacting mammalian ischemic tissue.

Figure 1

Figure 2A
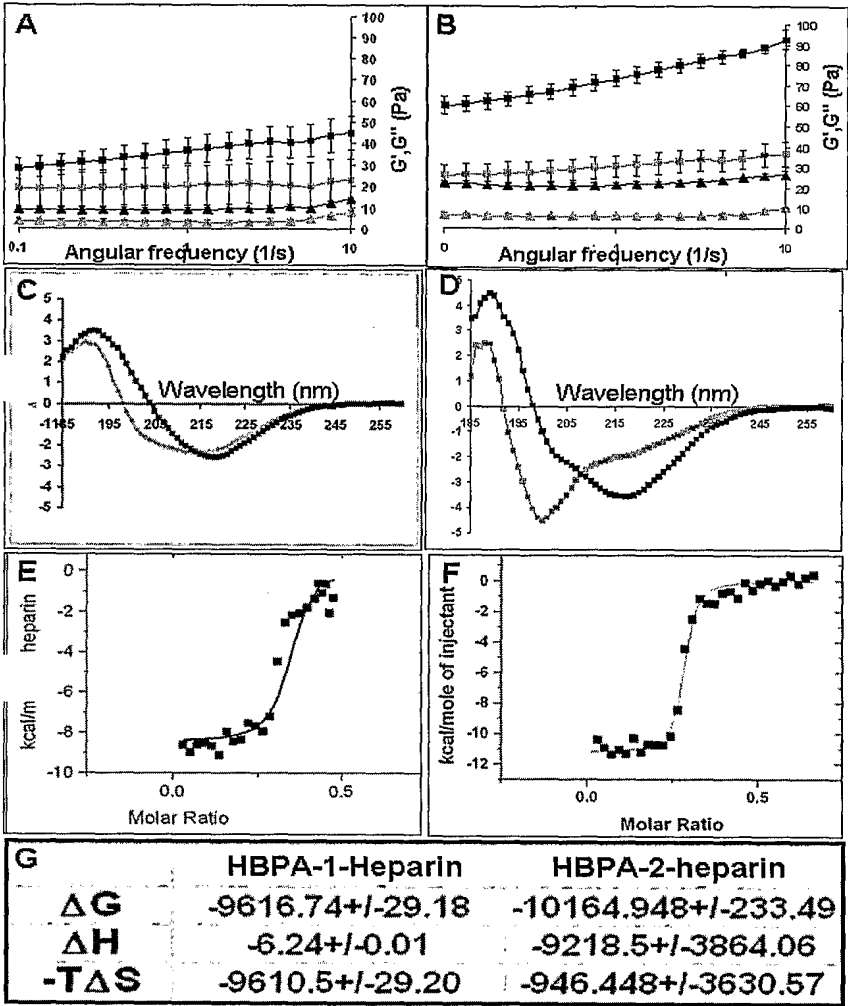

Figure 2C

Figure 2B

Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 3E

Figure 3F

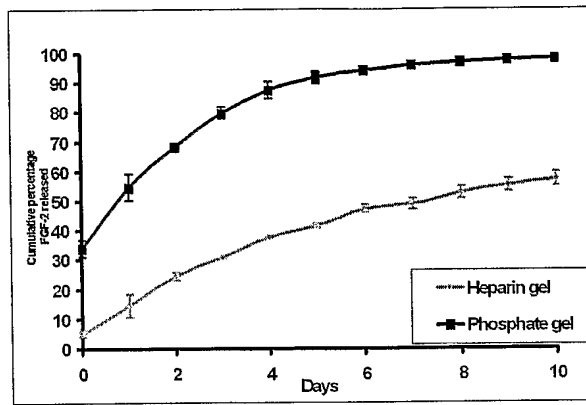| G	HBPA-1-Heparin	HBPA-2-heparin
ΔG	-9616.74+/-29.18	-10164.948+/-233.49
ΔH	-6.24+/-0.01	-9218.5+/-3864.06
-TΔS	-9610.5+/-29.20	-946.448+/-3630.57

Figure 3G

Figure 4

Figure 5A-H

Figure 6